Survival of *Salmonella enterica* and Shifts in the Culturable Mesophilic Aerobic Bacterial Community as Impacted by Tomato Wash Water Particulate Size and Chlorine Treatment

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ABSTRACT

Particulates of harvest debris are common in tomato packinghouse dump tanks, but their role in food safety is unclear. In this study we investigated the survival of *Salmonella enterica* and the shifts in relative abundance of culturable mesophilic aerobic bacteria (cMAB) as impacted by particulate size and interaction with chlorine treatment. Particulates suspended in grape tomato wash water spanned a wide size range, but the largest contribution came from particles of 3 to 20 µm. Filtration of wash water through 330 µm, applied after 100 mg/L free chlorine (FC) wash, reduced surviving cMAB by 98%. The combination of filtration (at 330 µm or smaller pore sizes) and chlorinated wash also altered the cMAB community, with the survivors shifting toward Gram-positive and spore producers (in both lab-simulated and industrial conditions). When tomatoes and harvest debris inoculated with differentially tagged *Salmonella* were washed in 100 mg/L FC for 1 min followed by filtration, only cells originating from harvest debris survived, with 85 and 93% of the surviving cells associated with particulates larger than 330 and 63 µm, respectively. This suggests that particulates suspended in wash water can protect *Salmonella* cells from chlorine action, and serve as a vector for cross-contamination.

KEYWORDS: Tomatoes; *Salmonella*; Chlorine wash; Particulate-association; Cross-contamination; Packinghouse

1. INTRODUCTION

Fresh produce is often consumed raw without a kill step for foodborne pathogens that may occasionally be present (Van Haute et al., 2015). While bacterial, viral and protozoan pathogens contaminating fresh and fresh-cut produce have all caused foodborne illness outbreaks, *Salmonella enterica* has been the major etiologic agent for illnesses associated with consumption of contaminated tomatoes (Bennett et al., 2015). While pathogen contamination occurred through multiple avenues, at least two outbreaks were attributed to packing house operations where warm tomatoes were placed in a cool, unchlorinated water bath (Sivapalasingam et al., 2004).

Flumes and dump tanks are used to wash tomatoes commercially, to remove soil, debris, and microorganisms from produce surfaces (Gombas et al., 2017; Zhou et al., 2014). Sanitizers and washing aids are added to the water to inactivate bacteria and improve the efficacy of foreign material removal, although reductions in bacterial populations on the produce rarely exceed 2 logs (Gil et al., 2009). Since bacteria released from contaminated produce into the aqueous phase are especially vulnerable to the sanitizer, major reductions in cMAB populations are achieved in the sanitized aqueous phase. Disinfection of the wash water is essential to avoid cross-contamination of microbial pathogens from contaminated to non-contaminated produce. (Gil et al., 2009; Luo et al., 2011; Van Haute et al., 2015; Gombas et al., 2017).

In aqueous conditions in the absence of particulate matter and without dissolved organic matter, *Salmonella* spp. are very susceptible to free chlorine (FC); 1 mg/L FC can reduce *Salmonella* spp. by 4 to 5 log in 5 s (Shen et al., 2013). However, reports also show that *Salmonella* cross-contamination occurred sporadically in water

containing high FC residuals. Rana et al. (2010) observed *Salmonella* cross-contamination in tomato wash water at or below 30 mg/L FC (2 positives out of 18 tomatoes), but not at 100 mg/L FC. Sreedharan et al. (2017) observed *Salmonella* cross-contamination, after enrichment of tomatoes previously washed in the presence of up to 50 mg/L FC (percentage of positive samples not reported), but in the absence of organic matter. In the presence of organic matter (from a soil source), cross-contamination was observed (one positive out of 36 tomatoes) even with 75 mg/L FC. Bolten et al. (2019) observed *Salmonella* cross-contamination in tomato wash water, a) between tomatoes in FC up to 50 mg/L (1 positive out of 180 samples), b) from inoculated debris to tomatoes in 50 mg/L FC (5 positives out of 180 tomatoes), c) at 100 mg/L FC (1 positive out of 60 tomatoes), and d) at 150 mg FC (1 positive out of 180 tomatoes). Historically, industrial washing of tomatoes has used high FC residuals, ranging from 50 to at least 150 mg/L (Zhou et al., 2014; Sreedharan et al., 2017). The current Florida, USA regulation requires 150 mg/L FC throughout their tomato packing operations (UFPA, 2018).

While many possible routes could lead to pathogen transfer, direct cross-contamination by bacterial cells that are unattached to particulates is unlikely the major contributor in these situations given the high FC concentrations. Thus, other possible mechanisms for these cross-contamination events may include: a) direct transfer when tomatoes contact each other; and b) particulate-mediated transfer when bacteria harbored by particulates survive chlorinated wash water and transfer to uncontaminated tomatoes.

Bacterial association with particulates can occur either by attachment, which involves adsorption (adherence), or by entrapment. Attachment can be active, through secretion of exopolymeric substances by the bacteria, or passive by physical

adsorption onto the particle (Simões et al., 2010). This association can increase the resistance of bacteria to sanitizer treatment by physically shielding the cells from sanitizers and/or reduce the diffusion of sanitizing chemicals (Örmeci and Linden, 2002; Dietrich et al., 2007; Lin et al., 2016; Mamane and Linden, 2006). While particulates are frequently found in tomato packinghouse dump tank wash water, and fresh-cut produce wash flumes, no studies have been published concerning the size distribution of these particulates. While the tomato industry food safety guidelines generally recommend removal of harvest debris (source of particulates) during tomato harvesting (UFPA, 2018), no reports are available regarding the effects of particulates on pathogen inactivation, cross-contamination, and food safety. Thus, the main objectives of this study were to 1) assess the size distribution of harvest debris particulates in tomato wash water; 2) determine the survival of *Salmonella enterica* and cMAB as impacted by particulate size and interaction with FC. Findings will illuminate the prevalence of particulate-mediated cross-contamination and provide a scientific basis for the development of science- and risk-based food safety regulation and industry standards.

2. MATERIALS AND METHODS

2.1. Tomatoes and inoculation

Grape tomatoes (*Lycopersicon esculentum* Mill) were obtained from a commercial tomato packing facility in Maryland, USA. Tomatoes, along with co-harvested plant materials (leaves, petioles, and stems, herein collectively termed “debris”), were collected and transported unwashed to the laboratory at Beltsville Agricultural Research Center, where they were stored at 12 °C for up to 24 h.

Six strains of *Salmonella* were used for this study, including three strains resistant to rifampin (strain-serovars: SL1344 Typhimurium; MDD314 Newport; USDA4559 Braenderup), referred as Sal-Rif^R, and three strains resistant to kanamycin (strain-serovars: FS3087 Typhimurium; SARA33 Heidelberg; SARB11 Derby), referred as Sal-Kan^R). All strains were obtained from collections at the Environmental Microbial and Food Safety Laboratory, USDA-ARS. Each strain was resuscitated from a glycerol stock, kept at -80 °C, by streaking onto Xylose Lysine Tergitol 4 agar (XLT4; Becton, Dickinson and Co., Sparks, MD, USA) supplemented with either 50 µg/ml rifampin or 50 µg/ml kanamycin, and subsequently grown from single colonies in Tryptic Soy Broth (TSB; Becton, Dickinson and Co.) supplemented with respective antibiotic at 42 °C with continuous shaking at 150 rpm. Cells were then concentrated by centrifugation at 4,500 g for 5 min, washed once in phosphate buffered saline (PBS; Becton, Dickinson and Co.), and re-suspended in PBS. The optical density of each strain was then adjusted to an OD_{600} of 0.3 by further dilution in PBS. Strains with Rif^R and those with Kan^R were used to prepare two separate inocula for differential and reciprocal application to grape tomatoes and debris to distinguish the source of the *Salmonella* during independent washing experiments.

Inoculation of tomatoes and debris was carried out as previously described (Bolten et al., 2019): Approximately 300 g of tomatoes (~10g/ea) without visible blemish were individually marked with a Sharpie marker, and immersed in 600 mL of inoculation cocktail for 5 min. Then, the tomatoes were air dried in a biosafety cabinet for 1 h at room temperature. For inoculation of debris, 3 g of debris was submerged in 15 mL of inoculation cocktail in a sterile filter bag (Whirl-Pak, Fort Atkinson, WI, USA) for 5 min, followed by draining excess inoculum and air drying for 1 h at room

temperature. Inoculated tomatoes and debris were then stored overnight at 12 °C prior to experimentation.

2.2. Tomato washing

Tomato wash water was prepared by submerging tomatoes and debris in tap water at a ratio of 1 kg/L for 30 min with agitation. After the removal of tomatoes and large pieces of debris using a sieve (pores of 0.5 x 2 cm), the resulting wash water was subjected to physicochemical analyses to determine chlorine demand and other water quality parameters. Sodium hypochlorite (Clorox, Aberdeen, MD, USA) was added to the wash water to achieve the desired free chlorine (FC) concentration. After 30 min stabilization, the free chlorine concentration was reassessed, and adjusted accordingly and the pH was adjusted to 6.5.

Tomato washing was carried out by washing approximately 300 g of grape tomatoes and 0.3 g of debris in 1 L wash water. For experiments with inoculated *Salmonella*, the 300 g of tomatoes consisted of 270 g of non-inoculated tomatoes and 3 tomatoes (~30 g) inoculated with *Salmonella*, and 0.3 g of debris inoculated with a different *Salmonella* cocktail than the tomatoes. Washing proceeded for 1 min with constant stirring at 25 °C.

2.3. Wash water sampling

Aliquots of wash water (50 mL) were sampled immediately at the end of washing periods and filtered either through a metal filter grid (1500 µm pore size), or a filter bag with 330 µm mesh openings (B01318WA, Whirl-Pak), or 63 µm mesh openings (BagPage F, Interscience, Gaithersburg, MD, USA). The filtrate was transferred to a sterile tube containing 1 mL of 10% sodium thiosulfate (Fisher Scientific, Frederick, MD, USA) to quench residual FC.

Wash water samples were also collected directly from the dump tank in the tomato packing facility where the tomatoes used in this study were obtained. Grape tomatoes were processed at a rate of 24,750 kg/h in a dump tank containing approximately 24,635 L of wash water. Three water samples were collected 20, 23 and 26 min after the washing process started, followed by filtering and quenching as done for the laboratory samples.

2.4. Wash water analyses

Appropriate dilutions of water samples in experiments without *Salmonella* inoculation or from the packing facility were plated on tryptic soy agar plates (TSA; Becton, Dickinson and Co.) and incubated at 30 °C for 48 h for to determine the count of cMAB . Dilutions of water samples from laboratory tomato washing experiments with *Salmonella* inoculation were plated on XLT4 agar containing 50 µg/ mL kanamycin or rifampin, and incubated at 42 °C for 24 h for *Salmonella* enumeration. Aliquots of 1 and 10 mL water samples were also filtered through 0.45 µm sterile membranes (Pall Laboratory, Nottingham, MD, USA) then incubated on respective agar plates to increase the detection range. In addition, enrichment for *Salmonella* was done by mixing 25 mL of sample with 25 mL double-strength TSB supplemented with 0.3 % sodium pyruvate, and incubating at 42 °C for 24 h, after which 4 µL droplets of enrichment culture were placed on both kanamycin- and rifampin-containing XLT4 plates and incubated at 42 °C for 24 h.

2.5. Microbial identification

For each water sample from washing without *Salmonella* inoculation, up to 25 colonies were picked from the TSA plates and subjected to genus identification by 16S rRNA gene sequencing. If a plate contained more colonies than required, a sector

of the plate was selected and all the colonies in that sector were picked to assure random selection. The selected colonies were inoculated into 200 µl of TSB on a 96-well microplate and incubated at 30°C for 48 h. After incubation, 10 µL of the culture was transferred to 90 µL of deionized water. This mixture was heated at 97 °C for 10 min and spun in a centrifuge at 4,000 rpm for 2 min, after which 4 µL of supernatant was added to 46 µL of PCR mix. The PCR mix consisted of 25 µL GoTaq Green Master Mix (Promega, WI, USA), 21 µL DNase free water containing 200 nmole/L forward and backward primers. Universal primers 27f (AGAGTTTGATYMTGGCTCAG, where Y is C or T and M is A or G) and 1492r (TACCTTGTTACGACTT) (Frank et al., 2008) were used to amplify the 16S rRNA gene sequences. PCR was conducted as follows: incubation for 5 min at 95 °C, followed by 35 cycles of i) denaturation for 1 min at 95 °C, ii) annealing for 1 min at 54 °C, and iii) extension for 2 min at 72 °C, and a final extension for 10 min at 72 °C. PCR product purification and sequencing was conducted by Eurofins Genomics (Louisville, KY, USA). For each isolate, sequences generated using forwards (27f) and reverse (1492r) primers were merged to generate a single query sequence for standard nucleotide BLAST analysis against NCBI database.

2.6. Physicochemical analyses of wash water

Chemical oxygen demand (COD), turbidity, total dissolved solids (TDS), and chlorine demand (CLD) were determined for the wash water. All measurements were conducted at room temperature (25 °C). COD was measured using the small-scale sealed-tube method (Hach, Loveland, CO, USA). Turbidity was measured with a turbidimeter (Orion AQ4500, Thermo Scientific, Waltham, MA, USA), TDS was determined with a TDS meter (135A Orion, Thermo Scientific). Chlorine demand was determined according to Teng et al. (2018) and Li et al. (2019).

Wash water particulate size distribution in the range of 0.2 and 1030 μm was profiled with a laser scattering particulate size distribution analyzer (Horiba Scientific, Edison, NJ, USA), with a 632.8 nm He-Ne laser and relative refractive index at 1.24 ± 0.00i. Surface area (rather than volume) of the particulates was selected as variable because attachment of bacteria would be more directly related to available surface area on the particulate. Larger sized particulates were characterized with a stereoscopic microscope (Olympus SZX12).

2.7. Statistical analyses

Statistical analyses were performed with R 3.5.1 (R-foundation). Normality of data and equality of variance among groups were tested with the Shapiro-Wilk and Levene's tests, respectively. Analysis of Variance (and Tukey HSD post-hoc test) was used to assess significant differences among groups. The Kruskal Wallis (and Conover-Iman post-hoc test) and Welch's unequal variances tests (and Games-Howell post-hoc test) were used instead of ANOVA when the assumptions of normality, or equality of variance were violated, respectively. Unless otherwise stated, P values <0.05 were considered statistically significant.

3. RESULTS

3.1. Wash water physiochemical properties and particulate size distribution

Tomato wash water, as well as the wash water used for other fresh-cut produce processing, carries large amounts of suspended solid materials. They consist of harvest debris, soils, and plant tissues from damaged products, and are collectively referred to as "particulates" herein. As shown in Figure 1A, particulate size distributions in the tomato wash water were strongly skewed toward the sub-visible or microscopic range, with over 90% of the total surface area represented by particulates

in the range of 3 to 20 µm. Filtrations at either 63 µm or 1500 µm did not alter the dominance of micrometer-sized particulates, although the proportion of sub-millimeter-sized particulates was greater with filtration at 1500 µm than at 63 µm. Figure 1B shows a representative microscopic view of particulate size distribution in unfiltered tomato wash water. In addition to the dominance of small particulates, irregularly-shaped particulates were commonly present. Multiple small particulates were observed to form aggregate structures, i.e., clusters visible as suspended solids without magnification by a microscope.

Filtration changed wash water turbidity, COD, and CLD, but with more pronounced effect on turbidity and COD (Table 1). Increasing in filtration strength by decreasing the filter pore size, progressively decreased turbidity and COD, with a filtration at 330 µm pore size achieving a 36% reduction in turbidity and a 44% reduction in COD. However, no substantial reduction in CLD was noted until the pore size was reduced to 63 µm, achieving 18% reduction in CLD.

3.2. Survival of cMAB in chlorinated tomato wash water

The potential for bacterial survival in chlorinated tomato wash water was first assessed by washing grape tomatoes along with harvest debris in wash water with different free chlorine concentrations, followed by water filtration at 330 µm. In the absence of FC, large bacterial populations (9 log CFU/mL) were observed in both filtered and unfiltered wash water (Figure 2). Increasing FC concentration from 0 to 5, 25, 50, and 100 mg/L progressively reduced bacterial survival in both filtered and unfiltered wash water. However, a significantly more pronounced reduction (ANOVA = 0.002) in cMAB was observed with filtration where at least 1.6 log lower cMAB was noted after filtration in each respective FC concentration.

The effect of particulate size on bacterial survival in chlorinated wash water was further explored with FC set at 0 and 100 mg/L. In the absence of FC, cMAB was very high (above 9 log CFU/mL), and filtration had no appreciable effect on cMAB reduction (Figure 3). On the other hand, after washing tomatoes in water containing 100 mg/L FC, cMAB counts were significantly reduced when filtered through 330 μm (Games-Howell = 0.003) and 63 μm (Games-Howell = 0.001), achieving 98%, and 99% reduction in counts of cMAB respectively, but not significantly when filtered through 1500 μm (Games-Howell = 0.098), achieving 64 % reduction in cMAB counts.

3.3. Genus level identification of surviving cMAB in chlorinated wash water

Genus identification of surviving bacteria in the chlorinated wash water was performed using 16s rRNA gene sequencing (Figure 4). The FC concentrations, filtration, and their interactions all played a role in bacterial species recovered. In the absence of FC and with 330 μm filtration, Gram-negative *Pantoea* and *Pseudomonas* predominated. In the 100 mg/L FC treatment, without filtration, the Gram-negative *Pseudomonas* were predominant, and after filtration with a large pore size (1500 μm), Gram-negative *Pseudomonas* and *Stenotrophomonas* represented a large part of the population. Filtration strength played a role in the composition of the surviving cMAB in chlorinated wash water when filtration through 330 and 63 μm was used. With filtration through 330 μm, the surviving bacterial population shifted toward Gram-positive genera, of which *Microbacterium* was the most abundant, and with 63 μm filtration, the population shifted toward predominately Gram-positive *Bacillus* and *Microbacterium*. Overall, in wash water that was unchlorinated, chlorinated but unfiltered, or chlorinated and filtered at 1500 μm, Gram-positive bacteria accounted for less than 20% of the total population, and the presence of sporeproducers was

below 2.5% (Figure 5). However, as the filter pore size decreased to allow more efficient removal of particulates, the prevalence of Gram-positive and spore-producers substantially increased. At the 330 µm cut-off, the Gram-positive genera accounted for 63.2% of the selected colonies, with 19.1% being spore producers. At 63 µm cut-off, the presence of Gram-positive and spore producers increased to 69% and 35%, respectively. These observations indicated that the survival of Gram-negative bacteria in chlorinated wash water was dependent on the presence of particulates within a certain size range.

3.4. Survival of bacteria in dump tank wash water from a commercial tomato packing facility

To determine whether the observed role of particulates in bacterial survival was also applicable to current industry operations, grape tomato dump tank wash water was obtained from a commercial packing house. The dump tank wash water had 96 ± 6 mg/L FC, 7.1 ± 0.1 pH, 861 ± 7 mV ORP, 99 ± 8 NTU turbidity, and 539 ± 45 mg/L COD. These parameter values are comparable to those of the simulated wash water that was used for laboratory experimentation (Table 1). Without filtration, the mean cMAB concentration was 5.3 ± 0.2 log CFU/100 mL. Filtration at 330 µm reduced cMAB concentration to 3.7 ± 0.2 log CFU/100 mL. In parallel with results obtained with wash water made in the laboratory, filtration shifted the bacterial community from primarily Gram-negative to Gram-positive and spore producers, with *Stenotrophomonas* and *Pseudomonas* predominating in unfiltered water and *Bacillus* and *Lysinibacillus* prevailing in filtered water (Table 2).

3.5. Effect of particulate size on *Salmonella* survival in tomato wash water

As established above, particulates strongly impacted the survival of Gram-negative bacteria in chlorinated wash water. Thus, additional studies were performed to evaluate the effect of particulates on the survival of *Salmonella*, a Gram-negative bacterium of significant food safety concern to the tomato industry (Figure 6). *Salmonella* strains with different antibiotic resistance markers (Sal-Rif^R and Sal-Kan^R) were used to reciprocally and differentially inoculate grape tomatoes and debris, yielding 8.1 and 7.9 log CFU/g Sal-Rif^R and Sal-Kan^R, respectively for debris, and 5.6 and 5.9 log CFU/g Sal-Rif^R and Sal-Kan^R, respectively for tomatoes (different levels of inoculation on tomatoes and debris are used to compensate for the different amount of tomatoes and debris used in the studies). In the absence of FC, washing either Sal-Rif^R inoculated tomatoes (~30 g) along with Sal-Kan^R inoculated debris (~0.3 g), or *vice versa*, resulted in 6-6.5 log CFU/100 mL Sal-Rif^R or Sal-Kan^R cells in the wash water (Figure 6). However, in the presence of 100 mg/L FC, only *Salmonella* cells originating from debris (Sal-Kan^R) were recoverable, while cells originating from tomatoes (Sal-Rif^R) were not recovered even after plating 10-fold more water samples on the same media (theoretic detection limit 1 log CFU/100 mL). However, when 25 mL of water samples were plated, 3 of 24 (1 unfiltered, 1 after 1500 µm filtration, and 1 after 63 µm) samples were positive for *Salmonella* that originated from tomatoes.

The recovery of debris-associated *Salmonella* in wash water was related to the applied filter pore size (Figure 6). *Salmonella* counts in 63 and 330 µm filtered water were significantly lower than that in unfiltered wash water (Tukey post-hoc: $p = 0.003$ and 0.02 respectively). On average, filtration through 1500 µm, 330 µm, and 63 µm pore sizes removed 50%, 85%, and 93% of surviving *Salmonella* cells, respectively.

4.0. DISCUSSION

4.1. Relationship between bacterial survival and particulates in chlorinated wash water.

Produce washing in chlorinated water is a common industry practice, often instituted to remove dirt and other foreign materials, and to reduce microbial contaminants, including foodborne pathogen populations (Gil et al., 2009). While free chlorine has limited efficacy for inactivating pathogens, the maintenance of a sufficient amount of free chlorine is required to prevent pathogen cross-contamination (Van Haute et al., 2015; Gombas et al., 2017).

Transference of bacterial cells from contaminated produce to a new surface in an aqueous milieu could occur by direct surface-to-surface contact, or via a process involving the bacterial cells releasing into the solution and reattaching to a new surface. In the presence of free chlorine, neither process is efficient, as most food-borne pathogens such as *E. coli* O157:H7 and *Salmonella* are highly susceptible to low concentrations of free chlorine in aqueous suspension (Shen et al., 2013). However, numerous studies also have shown that sporadic cross-contamination occurs at high FC concentrations and/or when no live pathogens are detected (Sreedharan et al., 2017; Bolton et al., 2019, Luo et al, 2011, 2012, and 2018). Thus, it is suspected that a third mechanism, i.e. particulates, serving as vectors for bacterial cross-contamination is in action.

In this study, we found major reductions (more than 3 log reduction) in cMAB in typical tomato wsh water, with less than 0.1% of bacterial cells surviving exposure to 100 mg/L FC (Figure 2). These surviving bacteria were likely to be physically associated with sub-millimeter particulates, as filtration through a 330 µm pore mesh removed over 98% of these cells, and no significant difference in survival was found

between 1500 µm and non-filtration, or between 330 µm and 63 µm pore mesh (Figure 2). Similar trends were observed when grape tomatoes and debris inoculated with *Salmonella* were washed in chlorinated water, in which filtration at 330 µm removed approximately 90% of the *Salmonella* that survived 100 mg/L for 1 min (Figure 6). Although particulate size distribution in a typical grape tomato wash water sample was such that particulates under 20 µm constituted over 90% of the total particulate surface, those under 330 µm seemed to play minimal roles in protecting bacterial cells from FC exposure.

Particulate size also substantially affected the relative abundance of cMAB. After chlorinated wash, removing particulates larger than 330 µm and especially larger than 63 µm reduced the presence of Gram-negative bacteria while selectively increasing the percentage of Gram-positive bacteria and spore producers (e.g. *Terribacillus* and *Bacillus*) in the surviving cMAB (Figures 4 and 5). This effect was more pronounced for water collected from a commercial packing facility, probably because repeated addition of chlorine during operations. Susceptibility of Gram-negative bacteria and resistance of Gram-positive bacteria to chlorine may have contributed to this differential survival.

4.2. Dependence of *Salmonella* survival and transference on particulates in chlorinated wash water.

This study also demonstrated an important role of harvest debris in *Salmonella* cross-contamination in the presence of a high chlorine concentration (100 mg/L FC, pH 6.5), since all positive samples were associated with debris inoculation. Given that in this study, 0.3 g of debris inoculated with 7.9-8.1 log CFU/g *Salmonella* and 30 g of tomatoes inoculated with 5.6-5.9 log CFU/g *Salmonella* were washed together, the

amount of *Salmonella* cells released from debris and tomatoes to water were comparable, as was confirmed by washing experiments in the absence of free chlorine (Figure 6). Thus, it is reasonable to infer that debris served a critical role in protecting *Salmonella* from chlorine inactivation action. Since debris contributed nearly exclusively to the generation of sub-millimeter particulates in a tomato washing system, this observation suggested that *Salmonella* survival in chlorinated wash water was dependent on attaching first (active, or passive through adsorption) to particulates. *Salmonella* survival due to entrapment within particles, but without previous attachment (adsorption) to a particle, is unlikely because *Salmonella* cells would have been in contact with the aqueous phase containing 100 mg/L FC, before entrapment. It also indicated that *Salmonella* transference from tomato to debris surface, and possibly *vice versa*, either by surface-to-surface contact or by releasing and then re-attaching was unlikely to occur in wash water containing 100 mg/L FC.

Several studies have examined *Salmonella* inactivation and cross contamination during simulated washing processes. Rana et al. (2010) found 3.7 log CFU/mL *Salmonella* in the wash water when washing inoculated mature green tomatoes without FC, but did not find *Salmonella* in the same wash water (detection limit 1 CFU/100 mL) when washing in 5, 30 and 100 mg/L FC, despite cross-contamination at 5 and 30 mg/L FC. Gereffi et al. (2015) simulated tomato wash water by using top soil to increase COD to a level found in commercial dump tank water. They observed that less than 1 log CFU/mL *Salmonella* survived after 2 s exposure to 10 mg/L FC at pH 6.5 and cross-contamination occurred under high COD (4000 mg/L COD) but not under low COD conditions. Using *Salmonella* strains tagged with different antibiotic markers, our recent studies demonstrated that *Salmonella* inoculated on debris was much more resistant to chlorine than those inoculated on tomatoes, and that cross-

405 contamination occurred from *Salmonella* cells originally inoculated on debris (Bolten
406 et al., 2019). However, since the total *Salmonella* cells released to the wash water was
407 higher from debris than from tomatoes due to their differing capacity to harbor
408 bacteria, the important role of debris in pathogen cross-contamination was inferred
409 but not proven. In the present study, comparable *Salmonella* cell populations were
410 released into wash water from both tomatoes and debris. The higher *Salmonella*
411 transference from inoculated debris than from inoculated tomatoes, demonstrates the
412 critical role of debris-generated particulates in pathogen cross-contamination (Figure
413 6).

414 In addition to its potential to generate large quantities of particulates, debris in
415 wash water also contributed greatly to the build-up of organic matter and chlorine
416 demand, which interferes with maintaining the desired FC residual (Zhou et al., 2014;
417 Van Haute et al., 2018; Teng et al., 2019). Therefore, excluding debris at harvest and
418 from entering into dump tanks may represent a great opportunity to reduce the
419 likelihood of *Salmonella* cross-contamination mediated by debris-derived particulates
420 during tomato washing. Currently, many packing houses apply a final rinse with fresh
421 water containing free chlorine, chlorine dioxide, or peroxyacetic acid. While this step
422 could, in theory, remove some bacteria-carrying particulates from tomatoes post-
423 dump tank washing, the effectiveness of this step for improving food safety needs to
424 be further examined. It worth noting that a high FC concentration at 100 mg/L was
425 selected because current Florida regulation requires 150 mg/L FC, and studies from
426 Sreedharan et al. (2017) reported that cross-contamination occurred in tomato wash
427 water with heavy organic matter also 100 mg/L FC was maintained. Given the
428 interaction of FC and filter pore size, additional studies are warranted to evaluate the

suitable FC residual and filter pore size for an effective removal of particulates to achieve food safety improvement.

5. CONCLUSIONS

In this study we examined the particulate size distribution in tomato wash water and its association with bacterial survival to free chlorine exposure, and *Salmonella* cross-contamination. Grape tomato wash water contains suspended particulates that are mostly formed from harvest debris (weeds, twigs, leaves, etc.). Their size ranges broadly, but dominate around 3 to 20 µm. Removing particulates greater than 330 µm had no impact on cMAB count in tomato wash water in the absence of chlorine, but significantly reduced the cell counts (by 98% or 1.6 log CFU/mL) in the wash water after 1 min exposure to 100 mg/L free chlorine. Filtration and chlorinated water wash also altered the surviving cMAB population. The population shifted to predominately Gram-positive and spore producers after 100 mg/L chlorine treatment followed by filtration at 330 µm, and especially at 63 µm. This was true for both wash water generated in the lab through simulated dump tank wash process, and collected directly from a commercial tomato packinghouse. When both tomatoes and harvest debris inoculated with *Salmonella* were washed in 100 mg/L for 1 min followed by filtration, only harvest debris-associated cells survived, with 85 and 93% of the surviving *Salmonella* cells associated with particulates larger than 330 and 63 µm, respectively. These results suggest that *Salmonella* cells in free suspension are readily inactivated by 100 mg/L, but those associated with particulates are protected. In other words, particulates from unwanted but often present harvest debris could serve as an important vector for pathogen cross-contamination, even at high chlorine concentration. Minimizing the harvest debris entering the tomato dump tank may represent an important action to mitigate pathogen cross-contamination.

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REFERENCES

Bennett, S. D., Littrell, K. W., Hill, T. A., Mahovic, M., Behravesh, C. B. 2015. Multistate foodborne disease outbreaks associated with raw tomatoes, United States, 1990-2010: a recurring public health problem. Epidemiol. Infect. 143, 1352-1359. https://doi.org/10.1017/S0950268814002167

Bolten, S., Gu, G., Luo, Y., Van Haute, S., Zhou, B., Millner, P., Micallef, S.A., Nou, X. 2019. *Salmonella* inactivation and cross-contamination on cherry and grape tomatoes under simulated wash conditions. Food Microbiol. 87, 103359. https://doi.org/10.1016/j.fm.2019.103359.

Camarillo, M.K., Loge, F.J., Darby, J.L., Ginn, T.R., Başağaoğlu, H., Foglia, L. 2011. Modeling the inactivation of microorganisms occluded in effluent wastewater particles to enhance operation of filtration and disinfection systems. Water Environ. Res. 83:313-325. https://doi.org/10.2175/106143010X12681059116851

476 Dietrich, J. P., Loge, F. J., Ginn, T. R., Başağaoğlu, H. 2007. Inactivation of particle-
477 associated microorganisms in wastewater disinfection: Modeling of ozone and
478 chlorine reactive diffusive transport in polydispersed suspensions. Water Res.
479 41, 2189-2201. https://doi.org/10.1016/j.watres.2007.01.038

480 Frank, J.A., Reich, C.I., Sharma, S., Weisbaum, J.S., Wilson, B.A. and Olsen, G.J.
481 2008. Critical evaluation of two primers commonly used for amplification of
482 bacterial 16S rRNA genes. Appl. Environ. Microb. 74(8): 2461-2470.
483 https://doi.org/10.1128/AEM.02272-07

484 Gereffi, S., Sreedharan, A., Schneider, K.R. 2015. Control of *Salmonella* cross-
485 contamination between green round tomatoes in a model flume system. J. Food
486 Protect.78, 1280-1287. https://doi.org/10.4315/0362-028X.JFP-14-524

487 Gil, M. I., Selma, M. V., López-Gálvez, F., Allende, A. 2009. Fresh-cut product
488 sanitation and wash water disinfection: Problems and solutions. Int. J. Food
489 Microbiol. 134, 37-45. https://doi.org/10.1016/j.ijfoodmicro.2009.05.021

490 Gombas, D., Luo, Y., Brennan, J., Shergill, G., Petran, R., Walsh, R., Hau, H.,
491 Khurana, K., Zomorodi, B., Rosen, J., Varley, R., Deng, K. 2017. Guidelines to
492 validate control of cross-contamination during washing of fresh-cut leafy
493 vegetables. J. Food Protect. 80, 312-330.https:/doi.org/10.4315/0362-028X.JFP-
494 16-258

495 Gurtler, J.B., Harlee, N.A., Smelser, A.M., Schneider, K.R. 2018. *Salmonella enterica*
496 contamination of market fresh tomatoes: A Review. J. Food Protect. 81, 1193-
497 1213. https://doi.org/10.4315/0362-028X.JFP-17-395

498 Li, J., Teng, Z., Zhou, B., Turner, E., Vinyard, B., Luo, Y. 2019. Dynamic changes of
499 physico-chemical properties of fresh-cut produce wash water as impacted by

commodity type and processing conditions. PLoS ONE 14(9), e0222174. https://doi.org/10.1371/journal.pone.0222174 Lin, T., Hou, B., Wang, Z. Chen, W. 2016. Inactivation of particle-associated *Escherichia coli* with chlorine dioxide. Water Sci. Technol.: Water Supply 17, 151-160. https://doi.org/10.2166/ws.2016.121

Luo, Y., Luo, Y., Nou, X., Yang, Y., Alegre, I., Turner, E., Feng, H., Abadias, M., Conway, W. 2011. Determination of free chlorine levels needed to prevent *Escherichia coli* O157:H7 cross-contamination during fresh-cut produce wash. J. Food Protect. 74, 352–358. https://doi.org/10.4315/0362-028X.JFP-10-429.

Luo, Y., Nou, X., Millner, P., Zhou, B., Shen, C., Yang, Y., Wu, Y., Wang, Q., Feng, H., Shelton, D. 2012. A pilot plant scale evaluation of a new process aid for enhancing chlorine efficacy against pathogen survival and cross-contamination during produce wash. Intl. J. Food Microbiol. 158, 133-139. https://doi.org/10.1016/j.ijfoodmicro.2012.07.008.

Luo, Y., Zou, B., Van Haute, S., Nou, X., Zhang, B., Teng, Z., Turner, E., Wang, Q., Millner, P.D. 2018. Association between bacterial survival and free chlorine concentration during commercial fresh-cut produce wash operation. Food Microbiol. 70, 120-128. https://doi.org/10.1016/j.fm.2017.09013

Mamane, H., Linden, K. 2006. Impact of particle aggregated microbes on UV disinfection. I: Evaluation of spore–clay aggregates and suspended spores. J. Environ. Eng.132, 596-606. https://doi.org/10.1061/(ASCE)0733-9372(2006)132:6(596)

522 Örmeci, B., Linden, K.G. 2002. Comparison of UV and chlorine inactivation of
523 particle and non-particle associated coliform. Water Sci. Technol.: Water
524 Supply 2, 403-410. https://doi.org/10.2166/ws.2002.0197

525 Poitelon, J.B., Joyeux, M., Welte, B., Duguet, J.P., Prestel, E., DuBow, M.S. 2010.
526 Variations of bacterial 16S rDNA phylotypes prior to and after chlorination for
527 drinking water production from two surface water treatment plants, J. Indust.
528 Microbiol. Biotechnol. 37, 117-128. https://doi.org/10.1007/s10295-009-0653-5

529 Ramey, B.E., Koutsoudis, M., Bodman, S.B., Fuqua, C. 2004. Biofilm formation in
530 plant–microbe associations. Curr. Opin. Microbiol. 7, 602-609.
531 https://doi.org/10.1016/j.mib.2004.10.014

532 Rana, S., Parisi, B., Reineke, K., Stewart, D., Schlesser, J., Tortorello, M., Fu, T.J.
533 2010. Factors affecting *Salmonella* cross contamination during postharvest
534 washing of tomatoes. 2010 IFSH Annual meeting. Institute for Food Safety and
535 Health, Illinois Institute of Technology, Chicago, Illinois. Available at:
536 https://www.researchgate.net/publication/266421667_Factors_Affecting_Salmo
537 nella_Cross-contamination_During_Postharvest_Washing_of_Tomatoes.
538 Accessed 02/01/2019

539 Ridgway, H.F., Olson, B.H. 1982. Chlorine resistance patterns of bacteria from two
540 drinking water distribution systems. Appl. Environ. Microbiol. 44, 972-987.

541 Shen, C., Luo, Y., Nou, X., Wang, Q., Millner, P.D. 2013. Dynamic effects of free
542 chlorine concentration, organic load, and exposure time on the inactivation of
543 *Salmonella*, *Escherichia coli* O157:H7, and non-O157 shiga toxin–producing *E.*
544 *coli*. J. Food Protect. 76, 386-393. https://doi.org/10.4315/0362-028X.JFP-12-
545 320

546 Simões, M., Simões, L.C., Vieira, M.J. 2010. A review of current and emergent
547 biofilm control strategies. LWT - Food Sci. Technol. 43, 573-583.
548 https://doi.org/10.1016/j.lwt.2009.12.008

549 Sivapalasingam, S., Friedman, C.R., Cohen, L., Tauxe, R.V. 2004. Fresh produce: A
550 growing cause of outbreaks of foodborne illness in the United States, 1973
551 through 1997. J. Food Protect. 67, 2342–2353.

552 Sreedharan, A., Li, Y., De, J., Gutierrez, A., Silverberg, R., Schneider, K.R. 2017.
553 Determination of optimum sanitizer levels for prevention of *Salmonella* cross-
554 contamination of mature round tomatoes in a laboratory model flume system. J.
555 Food Protect. 80, 1436-1442. https://doi.org/10.4315/0362-028X.JFP-17-032

556 Teng, Z., Van Haute, S., Zhou, B., Hapeman, K., Millner, P.D., Wang, Q., Luo, Y.
557 2018. Impacts and interactions of organic compounds with chlorine sanitizer in
558 recirculated and reused produce processing water, Plos ONE 13, 1-15.
559 https://doi.org/10.1371/journal.pone.0208945.

560 UFPA (United Fresh Produce Association). 2018. Food Safety Guidance for the
561 Tomato Industry. Available at https://www.fda.gov/media/129790/download.
562 Accessed on 01/06/2020.

563 Van Haute, S., Luo, Y., Sampers, I., Mei, L., Teng, Z., Zhou, B., Bornhorst, E.R.,
564 Wang, Q., Millner, P.D. 2018. Can UV absorbance rapidly estimate the chlorine
565 demand in wash water during fresh-cut produce washing processes? Postharvest
566 Biol.Technol. 142, 19-27. https://doi.org/10.1016/j.postharvbio.2018.02.002

567 Van Haute, S., Sampers, I., Jacxsens, L., Uyttendaele, M. 2015. Selection criteria for
568 water disinfection techniques in agricultural practices. Crit. Rev. Food Sci. 55,
569 1529-1551. https://doi.org/10.1080/10408398.2012.705360

570 Zhou, B., Luo, Y., Turner, E.R., Wang, Q., Schneider, K.R. 2014. Evaluation of
571 current industry practices for maintaining tomato dump tank water quality
572 during packinghouse operations. J. Food Process. Preserv. 38, 2201-2208.
573 https://doi.org/10.1111/jfpp.12200

574

575 **Figure Captions**

576 Figure 1. A: Particulate size distribution of tomato wash water (COD 665 ± 25 mg/L,
577 turbidity 127 ± 23 NTU), showing percentage surface area in function of particulate
578 size. B: Microscopic image (24X magnification) of particulates in unfiltered tomato
579 wash water.

580 Figure 2. The cMAB from tomato wash water (COD 892 ± 47 mg/L, turbidity 44 ± 7
581 NTU) after 1 min washing of 300 g tomatoes in 1 L wash water, containing 0 to 100
582 mg/L free chlorine (FC), pH 6.5, 25 °C, with or without 330 µm filtration (n=2).

583 Figure 3. The cMAB from tomato wash water (COD 665 ± 25 mg/L, turbidity 127 ± 23
584 NTU) after 1 min washing of 300 g tomatoes in 1 L wash water with 100 mg/L FC, pH
585 6.5, 25 °C and without filtration or with filtration through 1500, 330 or 63 µm filter
586 (n=6).

587 Figure 4. The cMAB community in tomato wash water subjected to chlorinated wash
588 and subsequent filtration.

589 Figure 5. Distribution of Gram-positive, Gram-negative, and spore producers in tomato
590 wash water subjected to chlorinated wash and subsequent filtration.

591 Figure 6. *Salmonella* counts in tomato wash water (COD 665 ± 25 mg/L, turbidity 127
592 ± 23 NTU) after 1 min washing in 0 or 100 mg/L FC, pH 6.5, 25°C; *Salmonella*
593 originating from tomatoes or debris (n=6).

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597 Table 1. Physicochemical properties of tomato wash water after filtration

Pore size (μm)	Turbidity (NTU)	Chemical Oxygen Demand (mg/L)	Chlorine Demand (mg/L)
No filter	127±22	665±25	201±4
1500	91±7	533±31	236±10
330	81±7	371±13	197±4
63	74±8	373±16	164±12

598 [1] Water collected after washing 1 kg of tomatoes with harvest debris in 1 liter tap
599 water for 1 min.

600

601 Table 2. Effect of filtration on shifts of culturable mesophilic aerobic bacterial genera
602 (cMAB) in tomato wash water¥ from a commercial packing facility

Genus	Gram	Spore producers	Unfiltered (n = 77)	Filtered (330 µm) (n=78)
Massilia	-	-	6.5£	
Pseudomonas	-	-	29.9	
Stenotrophomonas	-	-	41.6	
Leucobacter	+	-	2.6	
Bacillus	+	+	16.9	80.8
Lysinibacillus	+	+	2.6	19.2
All Gram+			22.1	100
All spore producers			19.5	100

£Number reported are percentage of total bacteria
¥ Wash water conditions: 96 mg/L free chlorine, pH 7.1, and 35 °F

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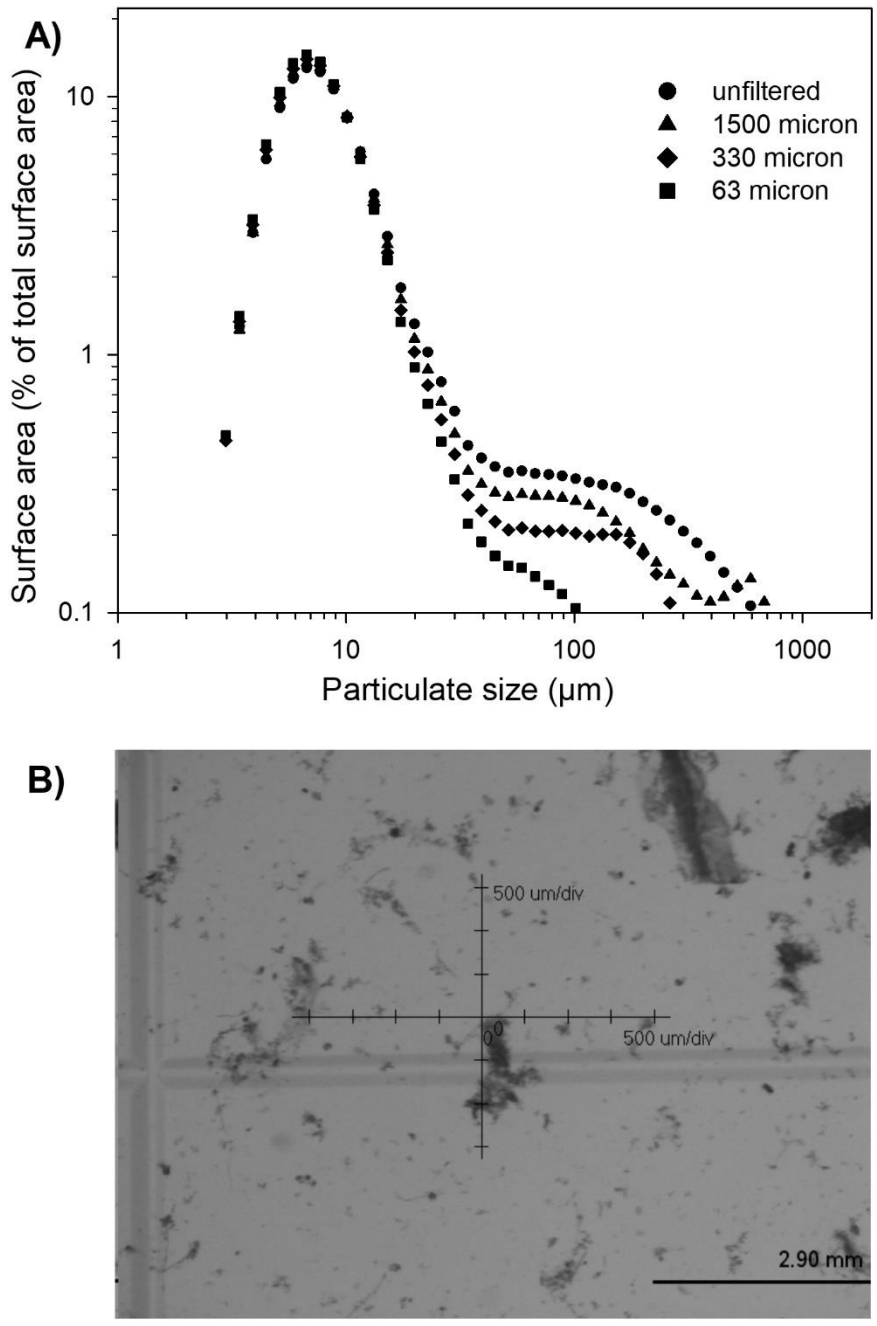
606 Figure 1.



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609 Figure 2.

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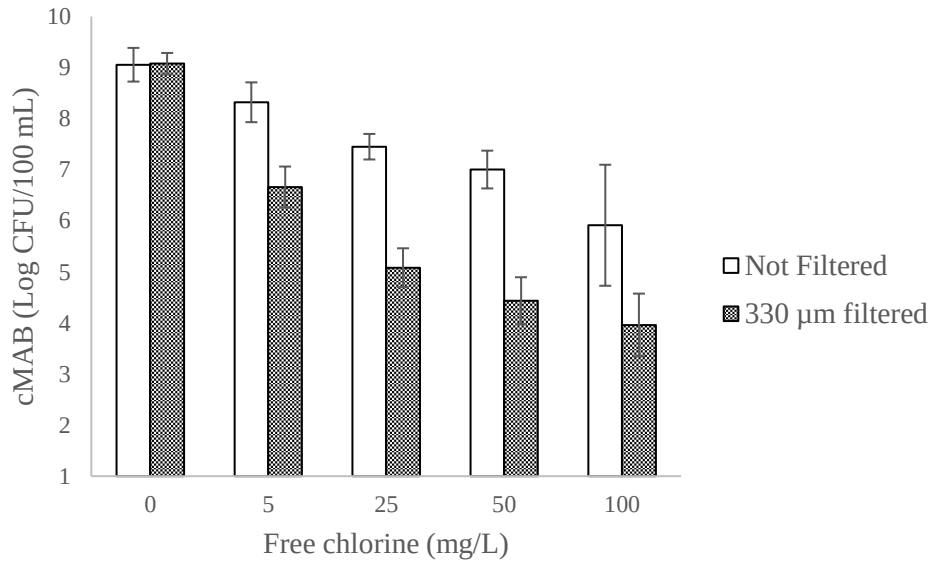

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613 Figure 3.

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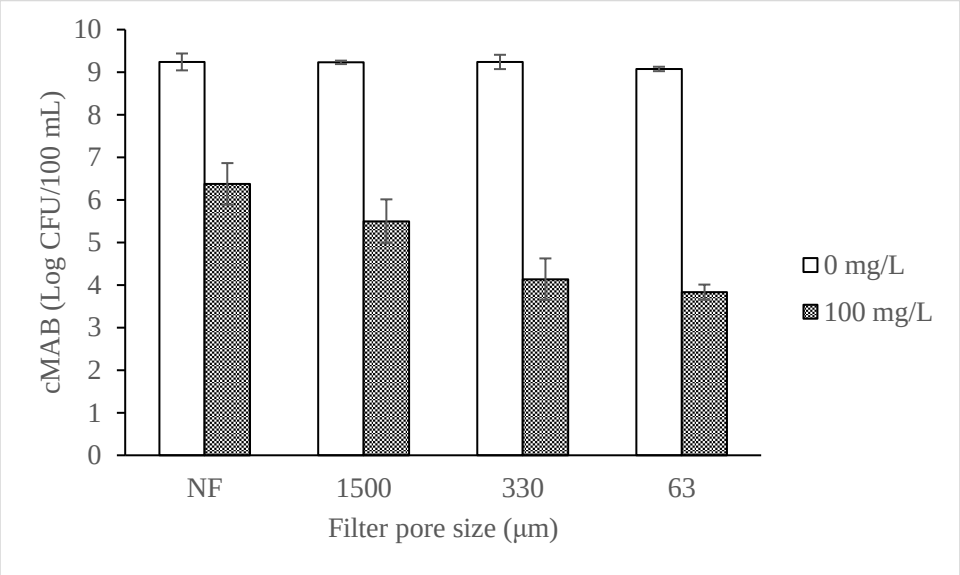

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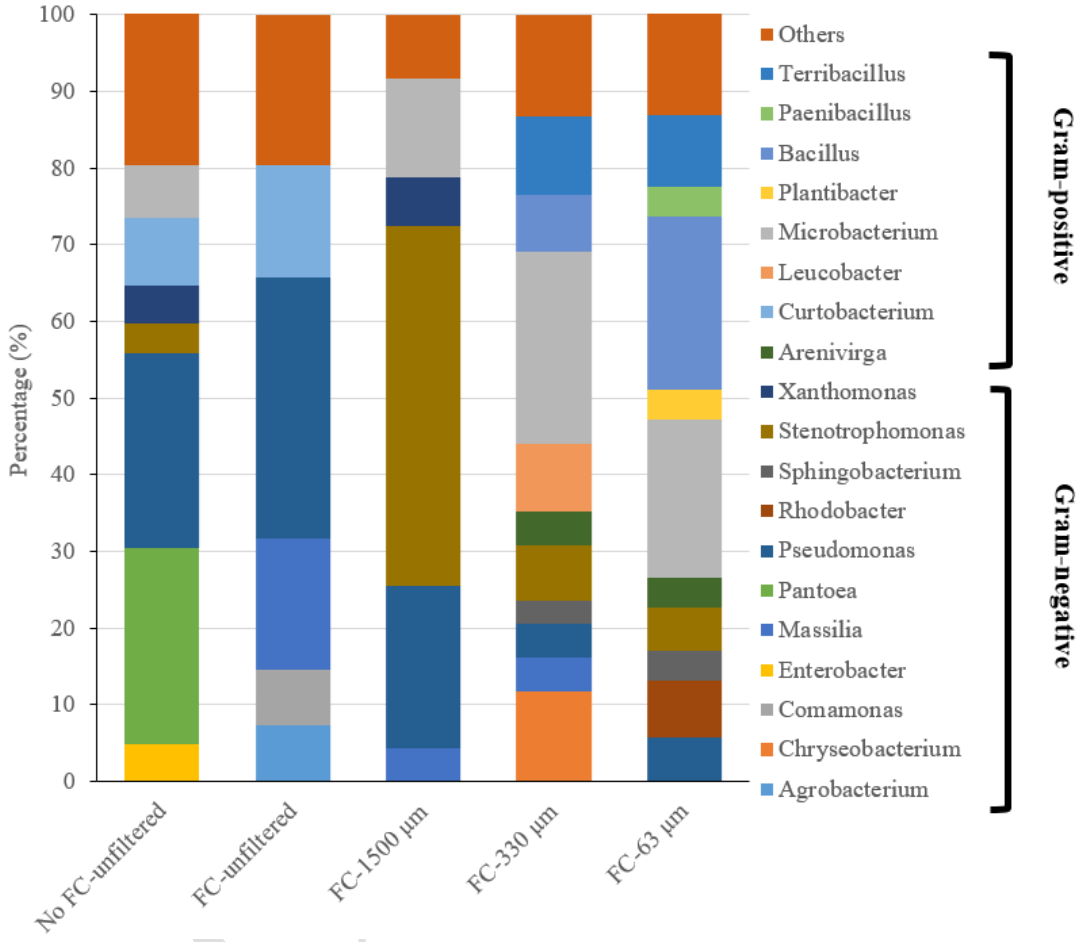
Figure 4.


100
90
80
70
60
50
40
30
20
10
0
Percentage (%)
No FC-unfiltered
FC-unfiltered
FC-1500 µm
FC-330 µm
FC-63 µm
Others
Terribacillus
Paenibacillus
Bacillus
Plantibacter
Microbacterium
Leucobacter
Curtobacterium
Arenivirga
Xanthomonas
Stenotrophomonas
Sphingobacterium
Rhodobacter
Pseudomonas
Pantoea
Massilia
Enterobacter
Comamonas
Chryseobacterium
Agrobacterium
Gram-positive
Gram-negative

624 Figure 5.

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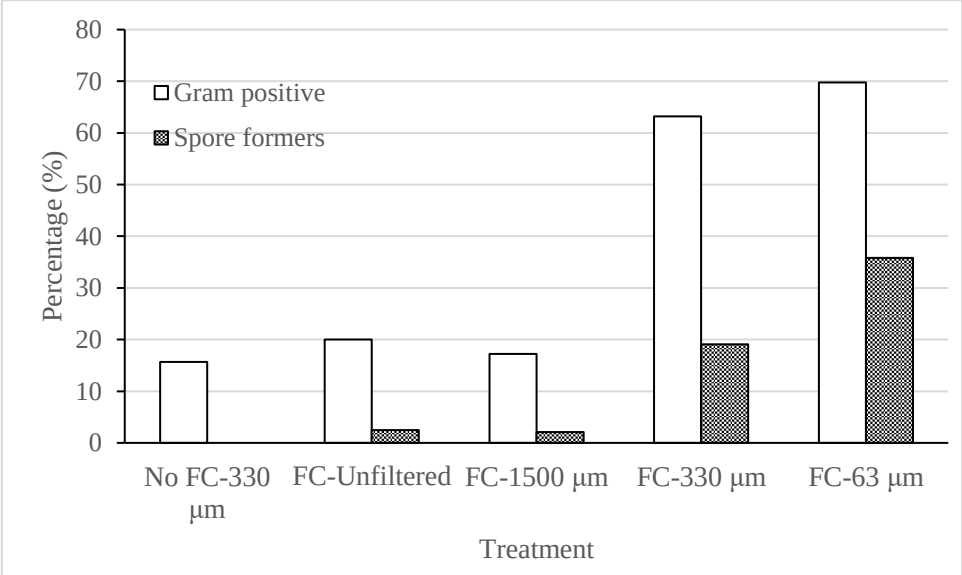

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629 Figure 6.

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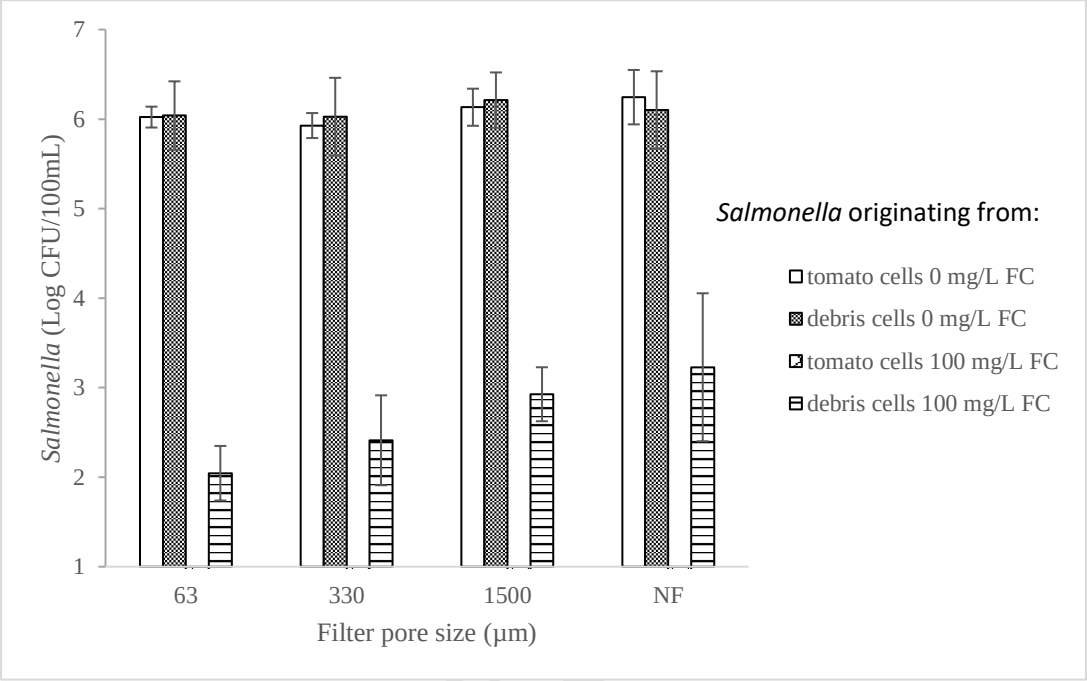

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